Calcium/calmodulin-dependent protein kinase I inhibits neuronal nitric-oxide synthase activity through serine 741 phosphorylation

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Abstract We demonstrate here that neuronal nitric-oxide synthase (nNOS) is phosphorylated and inhibited by a constitutively active form of Ca^{2+}/calmodulin (CaM)-dependent protein kinase I (CaM-K I1-293). Substitution of Ser^{741} to Ala in nNOS blocked the phosphorylation and the inhibitory effect. Mimicking phosphorylation at Ser^{741} by Ser to Asp mutation resulted in decreased binding of and activation by CaM, since the mutation was within the CaM-binding domain. CaM-K I1-293 gave phosphorylation of nNOS at Ser^{741} in transfected cells, resulting in 60–70% inhibition of nNOS activity. Wild-type CaM-K I also did phosphorylate nNOS at Ser^{741} in transfected cells, but either CaM-K II or CaM-K IV did not. These results raise the possibility of a novel cross-talk between nNOS and CaM-K I through the phosphorylation of Ser^{741} on nNOS.

Keywords: Neuronal nitric-oxide synthase; Calmodulin-dependent protein kinase I; Phosphorylation

1. Introduction

Ca^{2+}/calmodulin (CaM)-dependent kinase I (CaM-K I) is highly expressed in the brain and moderately in peripheral tissues [1]. It can phosphorylate a number of in vitro substrates, including synapsin 1 and 2 [2], cyclic AMP-response element binding protein (CREB) [3,4], and cystic fibrosis transmembrane conductance regulator (CFTR) [5]. But its physiological substrates are not clear. Neuronal nitric-oxide synthase (nNOS) is also a Ca^{2+}/CaM-dependent enzyme, which catalyzes the oxidation of L-arginine to generate nitric-oxide (NO) and L-citrulline [6]. It has been established that nNOS is phosphorylated by CaM-K II [7,8], leading to a reduction of nNOS activity through the phosphorylation at Ser^{847} of the enzyme [9,10]. It was recently shown that the Ser^{847} phosphorylation of nNOS occurs physiologically in rat hippocampus and hamster suprachiasmatic nuclei [11–13]. In transfected cells, CaM-K II but neither CaM-K I nor CaM-K IV can phosphorylate nNOS on Ser^{847} [14]. In the present study, we demonstrate that phosphorylation of nNOS at Ser^{741}, attenuating its enzyme activity, is catalyzed by CaM-K I, while CaM-K II and CaM-K IV are not involved.

2. Materials and methods

2.1. Materials

The cDNA for rat brain nNOS was a generous gift from Dr. Solomon H. Snyder (Johns Hopkins University School of Medicine, Baltimore, MD) [6]. A mouse anti-CaM-K IIα monoclonal antibody (mAb) was obtained from Oncogene. A mouse anti-CaM-K IV mAb was obtained from Transduction Laboratories, Inc. The mouse anti-nNOS mAb was obtained from Sigma. protein phosphatase 2A (PP2A) was purchased from Upstate Biotechnology. The rabbit anti-CaM-K I polyclonal antibody (pAb) was provided by Dr. Angus C. Nairn (Yale University School of Medicine, New Haven, CT) [15].

2.2. Anti-phosphopeptide-specific antibodies

Rabbit pAb raised against phosphopeptide based on the amino acid sequence of rat nNOS CEAVKF(Sp)AKLMG (NP741) was purified by tandem column chromatography using phosphopeptide and dephosphopeptide columns. NP847 pAb, recognizing phosphorylation at Ser^{847} on nNOS, was prepared as described previously [9].

2.3. Plasmid construction

PCWnNOS and pVL1393nNOS, the plasmids for the expression of nNOS in *E. coli* and Sf9 cells, respectively, were constructed as described before [16,17]. The pME18s-nNOS was generated as described previously [10]. CaM-K Iα, CaM-K IIα, CaM-K IV, and CaM kinase kinase (CaM-KK) were cloned from a rat brain cDNA library, then the cDNAs were cloned into pME18s. The constitutively active construct, pME18s-CaM-KK1-434, was generated as described previously [18]. CaM-kinase Iα1-293 and HA-CaM-K IV were subcloned into pME18s. The mutants CaM-K Iα1-293, K49A, nNOS S741A, S741D, and S847A, which are, respectively, substituted Lys^{49} by Ala, Ser^{741} by Ala, Ser^{741} by Asp, and Ser^{847} by Ala, were subcloned into pME18s and pCW. The nucleotide sequences of each mutant were confirmed.

2.4. In vitro activation of CaM-K Iα1-293 and CaM-K IV and phosphorylation of nNOS

CaM-K I1-293 is phosphorylated and activated by CaM-KK through Thr^{177} phosphorylation in both the presence and absence of Ca^{2+}/CaM [19]. We employed constitutively active form of CaM-KK (CaM-KK1-434) to activate CaM-K I1-293 in the absence of Ca^{2+}/CaM. Recombinant GST-CaM-kinase Iα1-293 [20] (5 μM) was preincubated with recombinant CaM-KK1-434 (190 nM) at 30 °C in 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 400 μg/ml bovine serum albumin, and 1 mM ATP. The reaction was

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Abbreviations: CaM, calmodulin; nNOS, neuronal nitric-oxide synthase; CaM-K I, II, and IV, calcium/calmodulin-dependent protein kinases I, II, and IV; CaM-KK, calcium/calmodulin-dependent protein kinase kinase; PP2A, protein phosphatase 2A

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terminated at 30 min by adding 7.5 mM EDTA and 5% glycerol at 4 °C. Recombinant CaM-K IV (3.8 μM) was preincubated with recombinant CaM-KK (168 nM) at 30 °C in 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1.2 mM $CaCl_2$, 1 μM CaM, and 1 mM ATP. The reaction was terminated at 30 min by a 10-fold dilution at 4 °C with 50 mM HEPES (pH 7.5), 5 mM EDTA, 2 mg/ml bovine serum albumin, and 10% glycerol. The phosphorylation reaction for nNOS was performed for 30 min at 30 °C in 10 μl of 40 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 0.4 mM DTT, 1 mM EGTA, 1 mM ATP, 400 μg/ml bovine serum albumin, and 1 μM nNOS in the presence of 100–200 nM of activated CaM-kinases with or without [γ-^{32}P]ATP. The lysates from rat brain were used as a source of protein phosphatase to dephosphorylate the phosphorylated nNOS. Briefly, rat brain was sonicated with 4 volume of 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM EGTA, 2 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin. After centrifugation at $15000 \times g$ for 15 min, the supernatant was used as a source of protein phosphatase.

2.5. Assay of nNOS activity

NOS activity was determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as described previously [16,21] in 20 μl of 40 mM Tris–HCl buffer, pH 7.5, containing 100 nM nNOS, 1 μM CaM, 100 μM L-[^{3}H]arginine, 0.1 mM NADPH, 20 μM BH_4, 4 μM FAD and 4 μM FMN.

2.6. Cell culture, transfection, and stimulation

HEK-293 cells in 10-cm dishes were transfected with pME18s plasmids containing wild-type nNOS (5 μg) with each combination containing CaM-K Iα (5 μg)/CaM-KK1-434 (1 μg), CaM-K IIα (5 μg), HA-CaM-K IV (5 μg)/CaM-KK1-434 (1 μg) using LipofectAMINE Plus method (Life Technologies, Inc.). After 36–48 h incubation, the cells were incubated for 60 min in serum-free medium. The cells were then stimulated with 10 μM A23187 (Sigma) for 3.5 min. For the determination of constitutively active form of CaM-K I that phosphorylates Ser^{741} of nNOS, the cells were transfected with pME18s plasmid containing wild-type nNOS or mutant nNOS (S741A) (5 μg), CaM-KK1-434 (1 μg), and CaM-K Iα1-293 or inactive CaM-K Iα1-293 (K49A) (5 μg).

3. Results and discussion

3.1. Effect of the phosphorylation of nNOS by a constitutively active form of CaM-K I on NOS enzyme activity

To assess the activity of phosphorylated nNOS by activated CaM-K I1-293, the conversion of [^{3}H]arginine to citrulline was measured. Phosphorylation of nNOS by activated CaM-K I1-293 decreased NOS activity to 10–15% (Fig. 1). Whether this attenuation was reversed by treatment of protein phosphatase, we used the extracts from rat brain as a source. The incubation of the phosphorylated nNOS with the extracts reversed the attenuation completely. The inclusion of 1 μM okadaic acid largely blocked the reversal effect by the extracts. The extracts could contain other factors that stimulate nNOS activity even in the presence of phosphorylation at Ser^{741}. Therefore, we examined whether treatment with PP2A resulted in the same effect with that of the extracts. As shown in Fig. 1, inset, the decrease in NOS activity induced by activated CaM-K I1-293 was reversed by treatment with PP2A.

3.2. Identification of CaM-K I1-293-dependent phosphorylation sites in nNOS

The CaM-binding ability of phosphorylated nNOS by activated CaM-K I1-293 was significantly blocked as assessed by the CaM overlay technique (Fig. 3A). Therefore, we made a single mutant by introducing Ala residues in place of Ser^{741} (S741A), since Ser^{741} was within the CaM-binding domain of nNOS. Wild-type and the mutant were expressed using the *E. coli* system and purified on 2′-5′-ADP-agarose as described in Section 2. The recombinant nNOSs were at least 90% pure as analyzed by densitometric scanning and gave a major band at 160 kDa on SDS–PAGE with Coomassie brilliant blue (CBB) staining (Fig. 2A). Phosphorylation of the mutant by activated CaM-K I1-293 was decreased as compared with that of the wild-type enzyme (Fig. 2B). The results suggest that activated CaM-K I1-293 may also phosphorylate nNOS at different sites as well, since the mutation of Ser^{741} did not fully block the phosphorylation of nNOS by the kinase. Next, we examined the ability of Ser^{741} residue phosphorylation to affect NOS activity. Mutation of Ser^{741} to Ala abolished CaM-K I1-293-dependent attenuation of NOS activity (Fig. 3B). Mutation of Ser^{741} into aspartic acid (S741D), to substitute for the negative charge afforded by the addition of phosphatase, mimicked the inhibition state by CaM-K I1-293 (Fig. 3B). The CaM-binding of phosphorylated nNOS or mutant S741D was almost completely blocked as assessed by the CaM overlay technique (Fig. 3A). Mutant S741A exhibited similar CaM binding ability as the wild-type enzyme and this was not significantly affected by the CaM-K I1-293-dependent phosphorylation of the mutant (Fig. 3A).

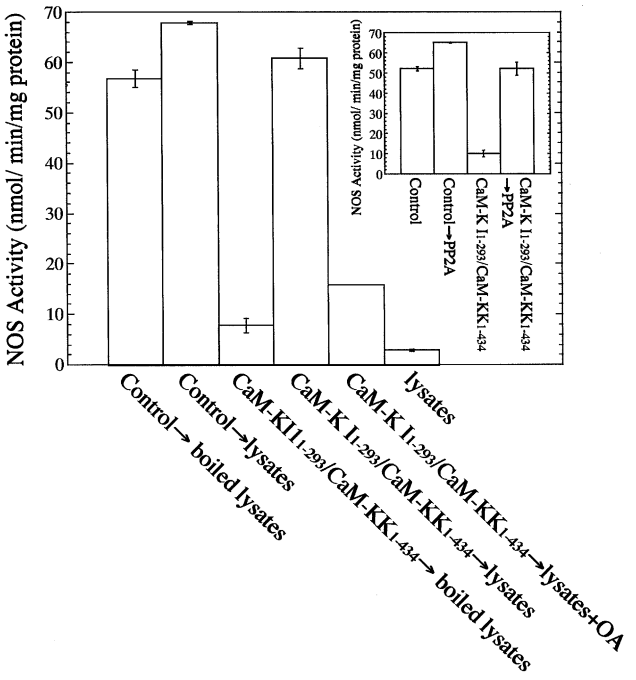

Fig. 1. Inhibition of nNOS activity by a constitutively active form of CaM-K I in vitro. Wild-type nNOS (1 μM) expressed in Sf9 cells was incubated with (CaM-K I1-293/CaM-KK1-434) or without (Control) an activated GST/CaM-K I1-293 (200 nM), as described under Section 2, for 30 min at 30 °C in the absence of Ca^{2+}/CaM with 1 mM ATP. Equivalent amounts (140 nM) of nNOS were treated either with heat-inactivated (10 min at 60 °C: boiled lysates) or non-heat inactivated (lysates) brain extracts (24 μg of protein) for 10 min at 30 °C in the presence or absence of 10 μM okadaic acid (OA). Then the NOS enzyme activity, negligible in the lysates itself, was assayed by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. The inset shows the data for an application of 100 nM PP2A instead of the extracts from rat brain. The means ± S.E. of three experiments are shown.

3.3. Phosphorylation of nNOS at Ser^{741} by CaM-K I1-293 in transfected cells

Since CaM-K I1-293-induced Ser^{741} phosphorylation in nNOS is functionally important for NOS activity, this residue

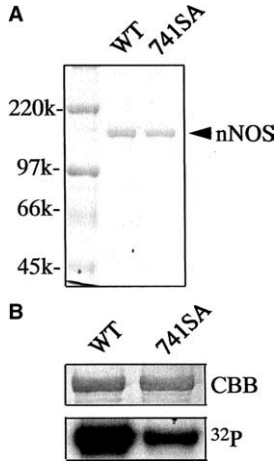

Fig. 2. Phosphorylation of wild-type and mutant nNOSs by a constitutively active form of CaM-K I in vitro. (A) Equal amounts (0.5 μg) of wild-type (WT) and the mutant nNOS, nNOS S741A (741SA) in *E. coli* were separated by 7.5% SDS–PAGE and stained by CBB. The *arrowhead* indicates the position of nNOSs. (B) 1 μg of wild-type (WT) or mutant nNOS, nNOS S741A (741SA) in *E. coli* was incubated at 30 °C with an activated GST/CaM-K I1-293, 100 μM [γ-^{32}P]ATP for 30 min in the absence of Ca^{2+}/CaM. The reaction was stopped by addition of sample buffer. Then, the samples were analyzed by SDS–PAGE on 7.5% gel and stained by CBB or autoradiography (^{32}P).

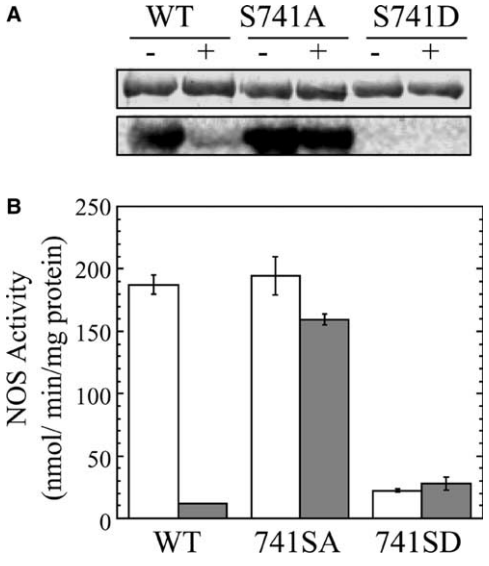

Fig. 3. Characterization of charged insertion mutant of nNOS. (A) Equal amounts (0.5 μg) of wild-type (WT) and the indicated mutants nNOSs in *E. coli* were incubated with buffer alone (−) or activated GST/CaM-K I1-293 for 30 min (+) at 30 °C in the absence of Ca^{2+}/CaM with 1 mM ATP. The reaction was stopped by addition of sample buffer. Then, the samples were analyzed by 7.5% SDS–PAGE, transferred onto PVDF membranes, and analyzed by reaction with biotinylated CaM overlay (*lower panel*). The same membranes were then stained with Amido Black (*upper panel*). (B) Wild-type and the indicated mutants nNOSs in *E. coli* were incubated with or without an activated GST/CaM-K I1-293 as in Fig. 1. Equivalent amounts (100 nM) of unphosphorylated (*opened column*) and phosphorylated (*closed column*) nNOSs were used for the NOS enzyme activity assay as in Fig. 1. The means ± S.E. of three experiments are shown.

can serve as a pertinent indicator to study in vivo nNOS phosphorylation by CaM-K I1-293 and/or wild-type CaM-K I. Thus, we prepared a rabbit polyclonal antibody (referred to as NP741), raised against the synthetic phosphopeptide: Cys–Glu–Ala–Val–Lys–Phe-phospho-Ser^{741}–Ala–Lys–Leu–Met–Gly. The specificity of NP741 was examined by Western blotting. NP741 reacted with nNOS phosphorylated by CaM-K I1-293 but not with non-phosphorylated nNOS (Fig. 4A). Phosphorylation of nNOS at Ser^{741} by CaM-K I1-293 was severely suppressed in the presence of Ca^{2+}/CaM (Fig. 4B). Fig. 4C shows that CaM at a 0.6:1 molar ratio to nNOS is required for half-maximal inhibition apparently. This result indicates that binding of Ca^{2+}/CaM to nNOS blocked the phosphorylation of nNOS at Ser^{741}. The activity of CaM-K I1-293 itself was not inhibited in the presence of Ca^{2+}/CaM, using syntide-2 as a substrate (data not shown). To examine whether CaM-K I1-293 can phosphorylate nNOS at Ser^{741} in cells, we analyzed the phosphorylation of nNOS at Ser^{741} in HEK-293 cells expressing nNOS and CaM-K I1-293. Cells were transfected with the wild-type nNOS and CaM-K I1-293/CaM-KK1-434 or inactive CaM-K I1-293 (K49A), Lys^{49} being an essential residue in the ATP binding motif [19]/CaM-KK1-434. We partially purified nNOS by 2′-5′-ADP-agarose affinity chromatography and quantified the phosphorylation state at Ser^{741} and protein expression level. Co-expression of CaM-K I1-293/CaM-KK1-434 resulted in significant enhancement in the phosphorylation of nNOS at Ser^{741} relative to cells co-expressing CaM-K I1-293 (K49A)/CaM-KK1-434 (Fig. 4D). Also, mutation of Ser^{741} to an Ala residue abolished CaM-K I1-293-dependent phosphorylation of nNOS. Therefore, we next examined whether the phosphorylation at Ser^{741} could directly influence the NOS activity in cells. The enzyme activity was determined by measuring the conversion of [^{3}H]arginine to citrulline using a partially purified nNOS from cell lysates on 2′-5′-ADP-agarose. Co-transfection of nNOS with CaM-K I1-293/CaM-KK1-434 resulted in decreased NOS activity in comparison to the cells co-expressing CaM-K I1-293 (K49A)/CaM-KK1-434 (Fig. 4D). Cells expressing mutant S741A nNOS also showed the decrease in NOS activity with CaM-K I1-293/CaM-KK1-434 but very little in comparison of the cells expressing wild-type nNOS.

3.4. *Phosphorylation of nNOS at Ser^{741} by CaM-K I in transfected cells*

To examine whether or not phosphorylation of nNOS at Ser^{741} by wild-type CaM-K I is observed in cells, nNOS was co-transfected with CaM-K I/CaM-KK1-434. Then, expressed nNOSs, partially purified by ADP-agarose affinity chromatography from lysates, were immunoblotted with NP741 either with or without 10 μM A23187 stimulation for 3.5 min. In the absence of stimulation, no phosphorylation was detectable. Stimulation of the cells with A23187 led to a remarkable increase in the nNOS phosphorylation (Fig. 5A). However, nNOS was not phosphorylated at Ser^{741} with A23187 stimulation in the cells expressing CaM- KIIα or HA-CaM-K IV/CaM-KK1-434. Meanwhile, phosphorylation at Ser^{847} with A23187 stimulation was significantly enhanced when CaM-KIIα was expressed. CaM-K II and CaM-K IV are known to be regulated by autophosphorylation and phosphorylation by CaM-KK, respectively, and retain partial activity even after full dissociation of Ca^{2+}/CaM [22]. The specificity of Ser^{741} residue phosphorylation was also examined by immunoblotting in vitro. Recombinant nNOS was phosphorylated by PKA, PKC, autophosphorylated CaM-K IIα, CaM-KK1-434 phosphorylated CaM-K IV or CaM-KI1-293 and analyzed by

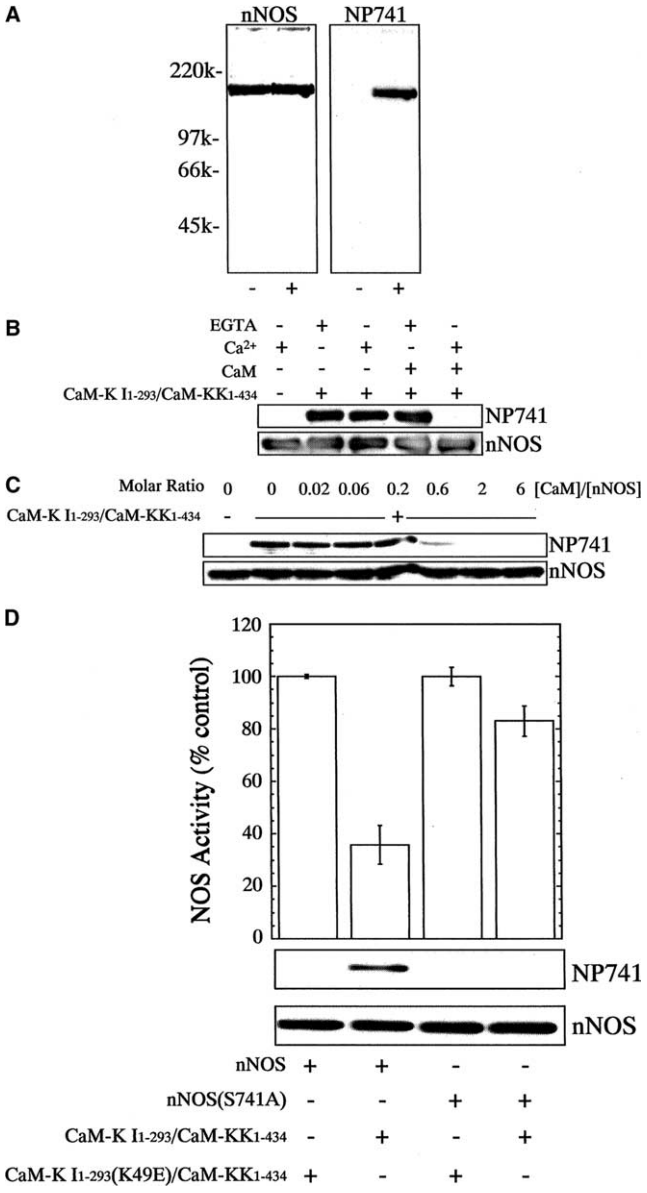

Fig. 4. Effects of a constitutively active CaM-K I-induced phosphorylation of nNOS at Ser^{741} in transfected cells. (A) 0.5 μg each of purified unphosphorylated nNOS (−) or phosphorylated nNOS (+) by activated GST/CaM-K I1-293 was separated by 7.5% SDS–PAGE, transferred onto PVDF membranes, and analyzed by Western blotting using an anti-nNOS antibody (*left panel*) or phosphospecific antibody, NP741 (*right panel*). (B) Equal amounts (0.5 μg) of wild-type nNOS in *E. coli* were incubated for 30 min at 30 °C either in the presence or absence of Ca^{2+}/EGTA/CaM/activated GST/CaM-K I1-293 (CaM-K I1-293/CaM-KK1-434) with 1 mM ATP. The reaction was stopped by addition of sample buffer. Then, the samples were analyzed by 7.5% SDS–PAGE, transferred onto PVDF membranes, and analyzed by Western blotting using an anti-nNOS antibody (nNOS) or phosphospecific antibody, NP741 (NP741). (C) nNOS (500 nM) was incubated for 30 min at 30 °C with CaM at 0, 10, 30, 100, 300, 1000, or 3000 nM, 1 mM $CaCl_2$, and activated GST/CaM-K I1-293 (CaM-K I1-293/CaM-KK1-434). Then, the samples were analyzed as in Fig. 2B. (D) 293 cells were transfected with combinations of expression plasmids of nNOS, nNOS S741A, CaM-K I1-293, CaM-K I1-293 K49E, and CaM-KK1-434. nNOSs were partially purified by ADP-agarose and subjected to Western blotting using an anti-nNOS antibody (*lower panel*) or phosphospecific antibody, NP741 (*middle panel*). Proportional amounts (1 μl of eluate) were subjected to NOS assay (*upper panel*). The means ± S.E. of three experiments are shown.

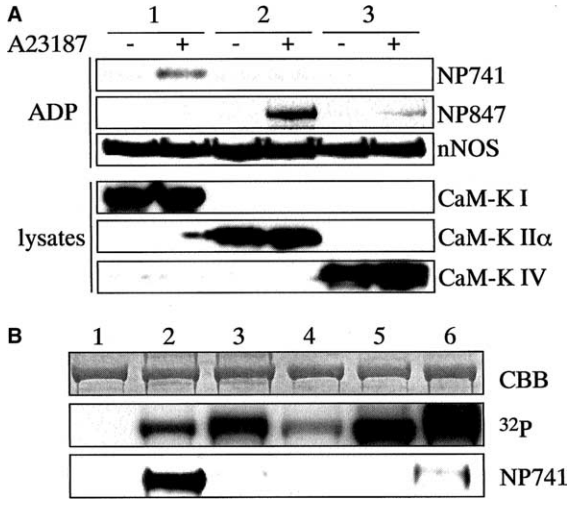

Fig. 5. Determination of kinases that phosphorylate Ser^{741} of nNOS. (A) 293 cells expressing nNOS with CaM-K I/CaM-KK1-434 (lane 1), CaM-K II (lane 2), or HA-CaM-K IV/CaM-KK1-434 (lane 3) were stimulated with buffer alone (−) or 10 μM A23187 for 3.5 min (+). Cell lysates were subjected to Western blotting with anti-CaM-K I (CaM-K I), anti-CaM-K II (CaM-K II), or anti-CaM-K IV (CaM-K IV) antibodies. Expressed nNOS was partially purified from transfected cells using ADP-agarose chromatography technique (ADP) and subjected to Western blotting with anti-nNOS (nNOS), NP741 (NP741), or NP847 (NP847). The data are representatives of at least two independent experiments. (B) 0.5 μg each of purified nNOS was incubated without any enzyme (lane 1), activated GST/CaM-K I1-293 (lane 2), autophosphorylated CaM-K II (lane 3), activated CaM-K IV (lane 4), PKA (lane 5), or PKC (lane 6) with or without [γ-^{32}P]ATP in the absence of CaM. After 7.5% SDS–PAGE, radioactive samples were stained with CBB and subjected to autoradiography (^{32}P). Non-radioactive samples were subjected to Western blotting using NP741 (NP741).

immunoblotting with NP741. NP741 reacted with nNOS phosphorylated by CaM-K I1-293 but not those by PKA, autophosphorylated CaM-K IIα, CaM-KK1-434 phosphorylated CaM-K IV. PKC did phosphorylate Ser^{741} residue, but only very slightly (Fig. 5B).

Our results support the notion that CaM-K I and CaM-K II modulate nNOS enzyme activity in cells by adding phosphate groups to Ser^{741} and Ser^{847} residues on the enzyme, respectively. Previously, it has been shown that introduction of a phosphate at Ser^{741} of CaM-binding peptide derived from nNOS prevents binding of CaM [23], consistent with our results. It was shown that endothelial NOS (eNOS) is phosphorylated at Thr^{495}, located in the CaM-binding sequence, by AMP-activated kinase and by PKC [24–26]. Phosphorylation of eNOS at this site reduces eNOS catalytic activity in vitro [24,26,27]. The position of Ser^{741} is part of a consensus site for protein phosphorylation (KXSXK) by PKC [28]. Indeed, phosphorylation of nNOS at Ser^{741} was induced by PKC in vitro. Although it was only very slightly compared to that by CaM-K I1-293 (Fig. 5B), it might be involved in the regulation of nNOS activity through Ser^{741} phosphorylation in cells. Although the Thr^{495} residue found in eNOS is not found in nNOS, the Ser^{741} residue in nNOS appears to represent an essential determinant for transducing the nNOS-CaM interaction into stimulation of enzyme activity via its phosphorylation. CaM-K II seems to exert its function mainly postnatally because its gene expression in the brain is evident

postnatally [29]. In contrast, CaM-K I may be intimately involved in neuronal differentiation in the brain, since the decrease in the expression of CaM-K I with the postnatal age is observed in most brain neurons [30]. Although a major concern is whether nNOS is actually phosphorylated at Ser^{741} in vivo, in the brain, this has not been shown. It needs to be demonstrated in future work whether nNOS can be phosphorylated at Ser^{741} by CaM-K I in vivo.

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